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Adiponectin promotes migration activities of endothelial progenitor cells via Cdc42/Rac1

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ABSTRACT

Adiponectin has anti-atherosclerotic effects through its direct actions on vascular cells. The present study investigates the molecular mechanisms of adiponectin in the migration of endothelial progenitor cells (EPCs) which play an important role in neovascularization and re-endothelization. The phosphorylation of Akt and the activations of Cdc42 and Rac1 were significantly increased by adiponectin. Adiponectin increased the migration activity of EPCs, which was completely inhibited by a PI3-kinase inhibitor. siRNA of Cdc42 or Rac1 completely inhibited the adiponectin-induced migration, but siRNA of Akt had no effects, indicating that adiponectin promotes the migration activities of EPCs mainly through PI3-kinase/Cdc42/Rac1.

Structured summary:
MINT-7217629: *PAK1* (uniprotkb:Q13153) *physically interacts* (MI:0914) with *CDC42* (uniprotkb:P60953) by *pull down* (MI:0096)
MINT-7217644: *PAK1* (uniprotkb:Q13153) *physically interacts* (MI:0914) with *Rac1* (uniprotkb:P63000) by *pull down* (MI:0096)

1. Introduction

Adiponectin is an adipocyte-specific secretory protein that has been implicated as a mediator of systemic insulin sensitivity with the liver and muscle as target organs [1–3]. The plasma adiponectin levels, which are decreased in obese patients, animal models of obesity, coronary artery disease and type 2 diabetes [4–6], correlate inversely with insulin resistance [7,8]. Adiponectin has also been recently revealed to have anti-atherogenic and anti-inflammatory properties. Overexpression of adiponectin reduces atherosclerotic lesions in mouse models, whereas adiponectin-deficient mice exhibit excessive intimal responses to vascular injury and diet-induced insulin resistance [9,10]. Moreover, adiponectin was found to be capable of stimulating angiogenesis [11]. Adiponectin promoted the formation of capillary-like structures from human umbilical vein endothelial cells (HUVECs) in vitro, functioned as a chemoattractant for HUVECs in migration and stimulated blood vessel growth in mouse [12].

On the other hand, endothelial progenitor cells (EPCs), which were first identified in adult peripheral blood mononuclear cells (MNCs) in 1997 [13], play an important role in postnatal neovascularization. EPCs contribute to the process of vasculogenesis, which comprises the adhesion of EPCs to the sites of vascularization and their subsequent infiltration and partial digestion of the target tissue resulting in the growth of a new blood vessel [14]. A number of experimental and clinical studies have revealed that transplantation of EPCs is an effective treatment for ischemic heart disease and arteriosclerosis obliterans because of the resulting neovascularization [15,16]. We revealed that transplantation of EPCs ameliorated diabetic neuropathy by increasing the tissue blood flow [17]. Many successful outcomes of experimental and clinical studies suggest that EPCs have a stronger potential for neovascularization than mature endothelial cells.

The mobilization and differentiation of EPCs were shown to be important in the process of adult neovascularization [18]. EPCs, cooperating with local endothelial cells, play a role in the formation

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of new blood vessels. Recent studies have provided evidence that the number and migratory activity of circulating EPCs inversely correlate with risk factors for coronary artery disease, suggesting that EPCs could serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk [19]. Furthermore, decreased numbers and functional deficits of EPCs were observed in types 1 and 2 diabetic patients [20,21], and the recruitment of EPCs into the aorta is decreased in diabetic animals. These results suggest that such deficits in the number and function of EPCs may contribute to the macrovascular complications in diabetic patients.

Although adiponectin is reported to increase the numbers and migration of EPCs [22,23], the precise mechanisms are still obscure. In this study, we have identified that adiponectin induces the migration of EPCs via the PI3-kinase/Cdc42/Rac1 pathway. Our findings reveal not only the role of adiponectin in neovasculogenesis and vascular repair, but also the therapeutic potency of adiponectin.

2. Materials and methods

2.1. Human umbilical cord blood

Human umbilical cord blood (50–120 ml) was obtained from each donor after childbirth. Written informed consent was obtained from all mothers before labor and delivery. Protocols for sampling human umbilical cord blood were approved by the Institutional Review Board.

2.2. Purification and cell culture of EPCs

EPCs were isolated from human cord blood using a Histopaque-density centrifugation method previously described [17]. Non-adherent cells were discarded after 48 h incubation. For the identification of EPCs, FACS analyses of freshly isolated cells were performed after 7 days in culture. Cells were fixed with 1% paraformaldehyde and labeled with phycoerythrin-conjugated CD31 (clone WN59; BD Biosciences, San Jose, CA); CD34 (clone 8G12; BD Biosciences, San Jose, CA); KDR (clone 89106; TECUNE Corporation, Minneapolis, MN) and Tie-2 (clone 83715; R&D Systems, Minneapolis, MN). Isotype-identical antibodies served as controls.

2.3. Identification of adiponectin receptor

The adiponectin receptor was identified by Western blot analyses using anti-human AdipoR1 antibody and anti-mouse AdipoR2 antibody (Alexis Biochemicals, San Diego, CA).

2.4. RNA interference

The siRNA-mediated knockdown of Akt, Cdc42 and Rac1 was performed using previously described methods [24]. The targeted sequences that effectively mediated the silencing of the expression of Akt, Cdc42 and Rac1 were prepared by Qiagen (Hilden, Germany). EPCs were transfected with the siRNAs or a 21-nucleotide irrelevant RNA (Qiagen) as a control, by using RNAifect (Qiagen) according to the manufacturer's protocol.

2.5. Western blot analyses

Confluent-grown EPCs were stimulated with 5 μg/ml full-length adiponectin (Biovender; Candler, NC) for the indicated periods. A sample (20 μg) of lysate protein was subjected to SDS–PAGE and detected by the first antibodies of anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK (Thr202/Tyr204) and anti-ERK (Cell Signaling Technology Inc., Beverly, MA).

2.6. Cdc42/Rac1 activity assay

To investigate whether adiponectin activates Cdc42 and Rac1, we used a Cdc42/Rac1 activation assay kit (from Cytoskeleton) according to the instructions provided by the manufacturer. This is a direct pull-down experiment performed by measuring the binding capability of GTP-bound Cdc42 or Rac1 to GSTPAK1 PBD fusion protein immobilized onto glutathione agarose beads. The amounts of active Cdc42 and Rac1 were measured by Western blot analyses.

2.7. Migration assay

To assess the migration of EPCs, we performed a modified Boyden chamber migration assay. The chambers were placed in 24-well dishes filled with M199 containing 0.1% BSA with adiponectin (5–10 μg/ml) or VEGF (10–20 ng/ml) added to the lower chamber, and the chambers were incubated for 12 h. After incubation, cells were labeled with Hoechist 33342. The adiponectin-stimulated migratory capacity was then quantified by counting the migrated EPCs on the lower surface of the filter using fluorescence microscopy.

2.8. Immunofluorescent staining of EPCs

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M PBS, permeabilized with 0.05%-triton X in PBS and stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen Co., Carlsbad, CA). Fluorescence was examined using a fluorescent microscope (BX51, Olympus, Tokyo, Japan).

2.9. Statistical analysis

All the group values were expressed as means ± S.E. Statistical analyses were assessed by one-way ANOVA. The level of significance was set at $P < 0.05$.

3. Results

3.1. Identification of EPCs

EPCs, isolated from the culture of cord blood-derived mononuclear cells, expanded from the attached cells (Fig. 1A). A linear formation was occasionally observed during the expansion of EPCs (Fig. 1B). More than 90% of the isolated cells were identified by DiI-acetylated LDL, which is a marker of vascular endothelial cells (Fig. 1C). On the other hand, HUVECs never showed the linear formation (data not shown). Flow cytometric analyses revealed positive staining with CD34 (81.3%), CD31 (89.4%), KDR (68.2%) and Tie-2 (78.8%) (Fig. 1D).

3.2. Identification of adiponectin receptor

To examine whether EPCs have adiponectin receptors, we evaluated the expressions of adiponectin receptors, AdipoR1 and AdipoR2 in EPCs using Western blot analyses. AdipoR1 was identified as a single band at 40 kDa (Fig. 2A). We also detected a single band stained with anti-mouse AdipoR2 (Fig. 2B), which had 93% homology with the human AdipoR1 sequences.

3.3. Adiponectin stimulates the phosphorylation of Akt, but not ERK

Adiponectin significantly stimulated the phosphorylation of Akt in a time-dependent manner (Fig. 3A). The phosphorylation of Akt was rapidly increased at 10 min after adiponectin stimulation. On

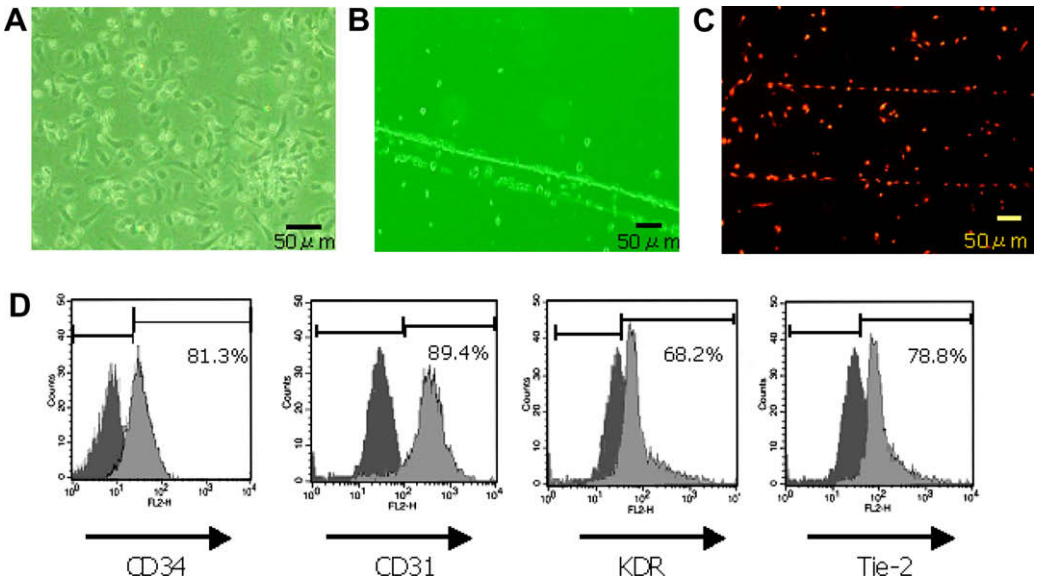

Fig. 1. Culture and identification of EPCs from cord blood. (A) EPCs, isolated from the culture of cord blood MNCs, expanded from attached cells. (B) A linear formation is occasionally observed during the expansion of EPCs. (C) The uptake of DiI-acetylated LDL. (D) Flow cytometric analysis revealed positive staining with CD34, CD31, KDR and Tie-2.

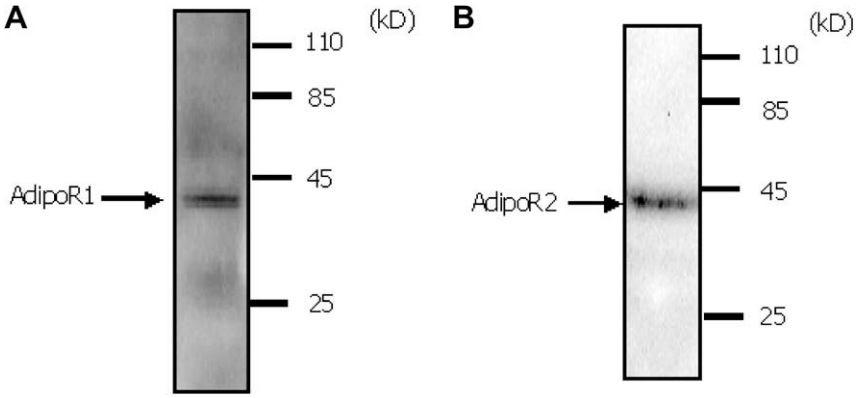

Fig. 2. Expression of adiponectin receptors, AdipoR1 (A) and AdipoR2 (B), in EPCs. Adiponectin receptors were identified by Western blot analyses using anti-human AdipoR1 antibody and anti-mouse AdipoR2 antibody.

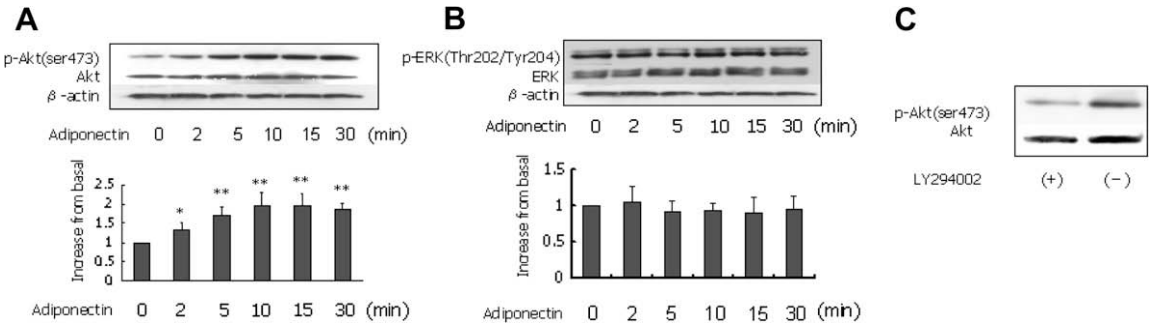

Fig. 3. The phosphorylations of Akt and ERK stimulated by adiponectin. Adiponectin stimulation (5 μg/ml) was conducted for the indicated time. (A) Adiponectin increased the phosphorylation of Akt. (B) The phosphorylation of ERK did not change by adiponectin. Results are shown as the means ± S.E. ($n = 3$) ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 min). One out of three experiments with similar results is shown. (C) The phosphorylation of Akt was inhibited by LY294002.

the other hand, adiponectin did not increase the phosphorylation of ERK (Fig. 3B). To assess whether adiponectin-stimulated Akt phosphorylation via PI3-kinase (PI3K), we measured the effect of LY294002, a PI3K inhibitor, on the phosphorylation of Akt. The phosphorylation of Akt was completely inhibited by LY294002 (Fig. 3C).

3.4. Adiponectin stimulates the activities of Cdc42 and Rac1

As shown in Fig. 4, both Cdc42 and Rac1 were activated by adiponectin stimulation. Maximum Cdc42 and Rac1 activation occurred at 2 min after adiponectin stimulation (Fig.4A and B). Next, we measured the effect of LY294002 on the activation of Cdc42 and

Rac1. Both Cdc42 and Rac1 were inhibited by LY294002, indicating that adiponectin-stimulated Cdc42 and Rac1 via PI3K (Fig. 4C).

3.5. Adiponectin promotes migration activity of EPCs via PI3K/Cdc42/Rac1

The number of migrated cells attracted by adiponectin was about seven times greater than that under the serum-free condition (Fig. 5A). The migratory activities of EPCs were increased in a dose-dependent manner by adiponectin as well as by VEGF. The adiponectin-induced enhancement of migratory activities was completely inhibited by LY294002 (91.7% suppression) (Fig. 5B). On the other hand, the MEK inhibitor, PD98059 did not inhibit the adiponectin-induced EPC migration, suggesting that an ERK-dependent pathway is not involved in the migratory effect of adiponectin in EPCs.

To elucidate the signaling pathway of adiponectin-stimulated migration, targeted knockdown using siRNA was performed. Western blot analyses showed that transfection with the Cdc42, Rac1 and Akt siRNAs effectively reduced the expression levels by over 90% (Fig. 6A). As shown in Fig. 6B, the adiponectin-induced migratory activities were completely inhibited by siRNA of Cdc42 and Rac1 (85.7% suppression by Cdc42-siRNA and 88.5% suppression by Rac1-siRNA). However, siRNA of Akt had no effect on the adiponectin-stimulated migration (1.4% suppression). These observations suggest that adiponectin stimulates Akt, Cdc42 and Rac1 via PI3K, but the migratory effects of adiponectin on EPCs mainly act through PI3K/Cdc42/Rac1 (Fig. 7).

3.6. Adiponectin promotes actin organization of EPCs via Rac1

The actin structure was visualized by staining EPCs with Alexa Fluor 488-conjugated phalloidin, which probes filamentous actin. EPCs without stimulation did not form clear actin organization (Fig. 8A). Adiponectin induced actin organization and lamellipodia formation at the cortex of EPCs (Fig. 8B). Rac1-siRNA blocked adiponectin-induced actin organization and lamellipodia formation in EPCs (Fig. 8C). On the other hand, Akt-siRNA did not effect adiponectin-induced actin polymerization and lamellipodia formation in EPCs (Fig. 8D).

4. Discussion

EPCs contribute to the process of vasculogenesis, which comprises the adhesion of EPCs to the sites of vascularization and their subsequent infiltration and partial digestion of the target tissue, resulting in the growth of a new blood vessel. In the present study, we have reported, for the first time, that adiponectin at its physiological plasma concentration could promote EPC migration through PI3K/Cdc42/Rac1.

Many successful outcomes of experimental and clinical studies suggest that EPCs have a stronger potential for neovascularization than ECs. We reported that transplantation of EPCs ameliorated diabetic neuropathy by increasing the tissue blood flow [17]. Enhanced cell migration is one of the underlying mechanisms in angiogenesis of EPCs. Therefore, inducing EPCs migration is considered to be a potentially effective strategy for angiogenesis. On the other hand, decreased numbers and functional deficits of EPCs in diabetic patients have been reported. In both types 1 and 2 diabetic patients, the numbers of EPCs were significantly decreased compared with normal subjects, and this was inversely correlated with HbA1c [20,21]. Tube formation of EPCs was also decreased in types 1 and 2 diabetic patients. In vitro experiments revealed that high glucose decreased the proliferation of EPCs [25]. These results suggest that the impaired function of EPCs may contribute to the diabetic macroangiopathy.

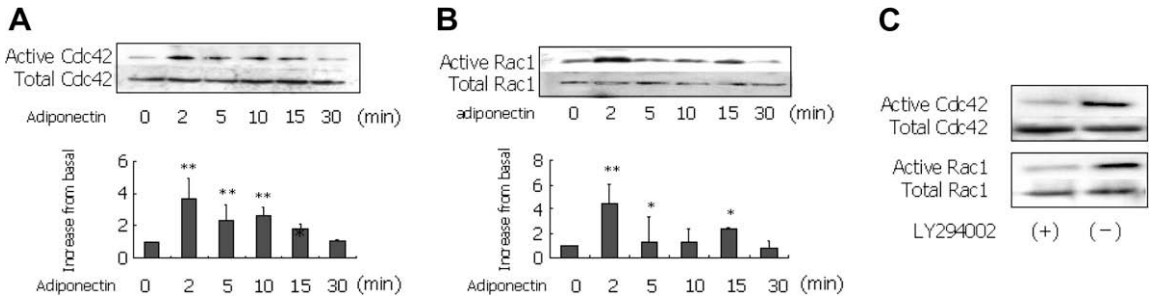

Fig. 4. The activations of Cdc42 and Rac1 stimulated by adiponectin. Adiponectin stimulation (5 μg/ml) was conducted for the indicated time. The activations of Cdc42 and Rac1 were detected by pull-down assay. (A) Adiponectin stimulated the activation of Cdc42. (B) Adiponectin stimulated the activation of Rac1. Results are shown as the means ± S.E. ($n = 3$) ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 min). One out of three experiments with similar results is shown. (C) Both Cdc42 and Rac1 were inhibited by LY294002.

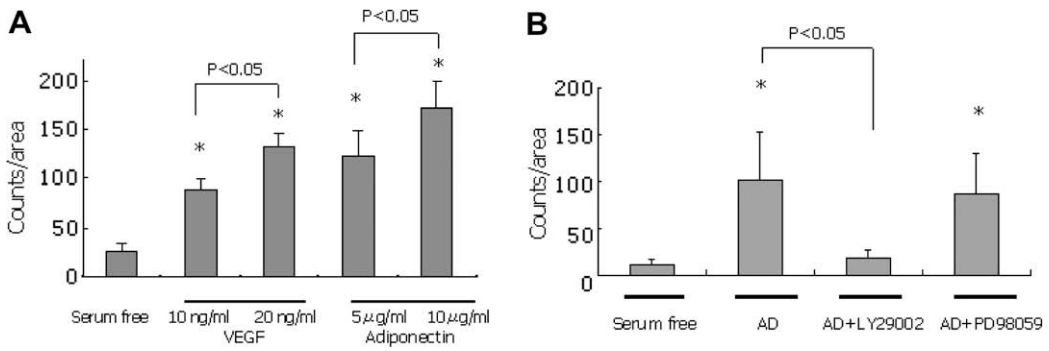

Fig. 5. Migration of EPCs stimulated by adiponectin. (A) Modified Boyden chamber assay was performed with adiponectin or VEGF as chemoattractant. (B) Adiponectin-induced enhancement of migration activities was inhibited completely by LY294002, but not by PD98059 ($^{*}P < 0.05$, vs. serum free control).

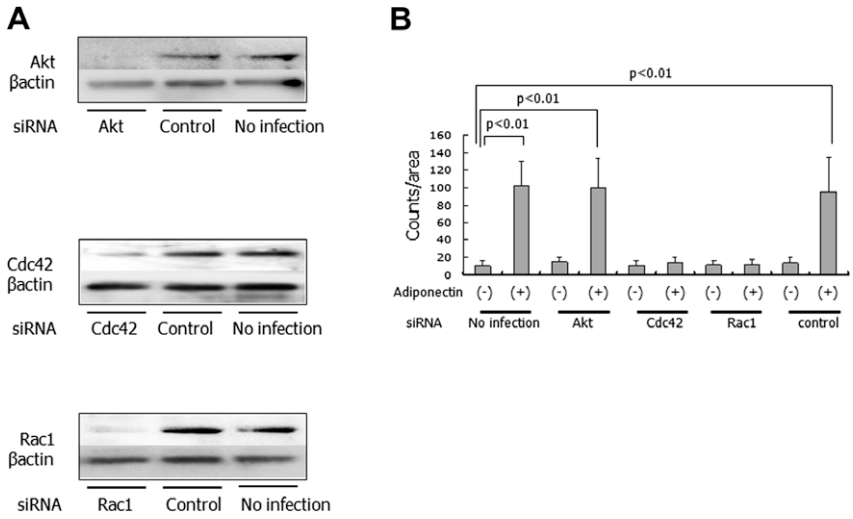

Fig. 6. The effects of Akt, Cdc42 and Rac1 on adiponectin-induced migration of EPCs. (A) The siRNA-mediated knockdown of Akt, Cdc42 and Rac1. (B) Adiponectin-induced migration activities were completely inhibited by siRNA of Cdc42 and Rac1 (85.7% suppression by Cdc42-siRNA and 88.5% suppression by Rac1-siRNA). However, the migration activity was not inhibited by a siRNA of Akt (1.4% suppression). Results are shown as the means ± S.E. ($n = 3$).

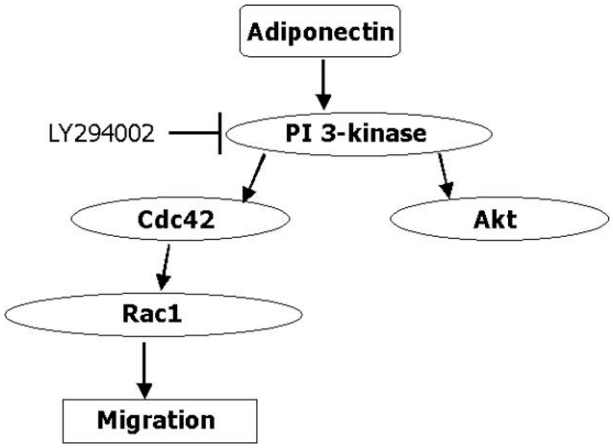

Fig. 7. Proposed scheme for adiponectin-stimulated signaling in the migration of EPCs.

Adiponectin was found to be capable of stimulating angiogenesis. Adiponectin also potently increased both the EPC numbers and migration capability [22,23]. Another report revealed that adiponectin knockout mice had a primary state of endothelial dysfunction with increased leukocyte–endothelium adhesiveness [26]. The present study confirmed the promotion of the migration of EPCs by adiponectin. PI3K, which is upstream of Akt and Cdc42, is one of the most important regulatory proteins involved in controlling several key functions of the cell, such as cell growth, aging, and transformation [27,28]. It was demonstrated in this study that adiponectin caused a dose-dependent migration of EPCs. This adiponectin-mediated migration was markedly suppressed by the administration of a PI3K inhibitor, LY294002. These results indicate that the effect of adiponectin on the EPC function was mediated in a PI3K-dependent manner.

Akt is an important regulator of various cellular processes including glucose metabolism and cell survival. Akt has been reported to directly activate eNOS, suggesting that Akt may regulate the increased production of nitric oxide in response to adiponectin stimulation [29,30]. Adiponectin has recently been revealed to stimulate the PI3K/Akt signaling pathway to enhance EC proliferation and migration [12]. However, in this study, we found that the activation of Akt was not essential for the adiponectin-induced migration of EPCs. Conflicting reports exist as to the role of Akt in cell migration. Several studies have demonstrated that Akt plays a positive role in migration, whereas other studies have reported conflicting results [31–33]. The discrepancy in the results, obtained in response to different stimulators, could be due to the activation of different signaling pathways in the different cell types.

The Rho family GTPases play critical roles in various situations such as cell growth, metastasis, morphogenesis, organogenesis and pathogenesis [34]. Cdc42 and Rac1 were reported to act as downstream effectors of PI3K in several growth factor-stimulated pathways [35,36]. We have discovered that Cdc42 and Rac1 were essential downstream proteins in EPC migration. Migrating cells cause a special actin organization, called "lamellipodium", at the leading edge of migration [37]. We have shown that adiponectin stimulates the lamellipodium formation at the cortex of EPC, which is suppressed by the inhibition of Rac1. These data suggest that the pro-angiogenic effects of the adiponectin-stimulated PI3K activity are due in large part to an activation of Cdc42/Rac1.

On the other hand, adiponectin did not affect the phosphorylation of ERK1/2. We also observed that the MEK inhibitor, PD98059, did not inhibit the adiponectin-induced EPC migration suggesting that none of those kinase-dependent pathways is involved in the migratory effect of adiponectin. Our data are consistent with a previous report that adiponectin affected no phosphorylation of ERK induced by TNF-α in HAEC.

Adiponectin in the circulation forms a complex of a trimer (high molecular weight form). We used the full-length adiponectin comprised of both the high and low molecular weight forms. We found that EPCs have both adiponectin receptor types 1 and 2 (AdipoR1 and R2). These results provide important insights into the molecular mechanisms underlying the promotion of EPC migration by adiponectin. However, we were not able to elucidate whether it was adiponectin receptor R1 or R2 that contributes to the EPC migration induced by adiponectin. Further study is being conducted in our laboratory to answer this question.

In conclusion, we revealed that adiponectin at the physiological plasma concentration can promote the migration of EPCs, mainly through PI3K/Cdc42/Rac1. Our present study provides important insights into the molecular mechanisms underlying EPC migration. Taken together, these results suggest that exogenous supplementation of adiponectin could be useful for therapeutic angiogenesis in patients who suffer from vascular complications such as ischemic heart disease and diabetes. This study may provide a better

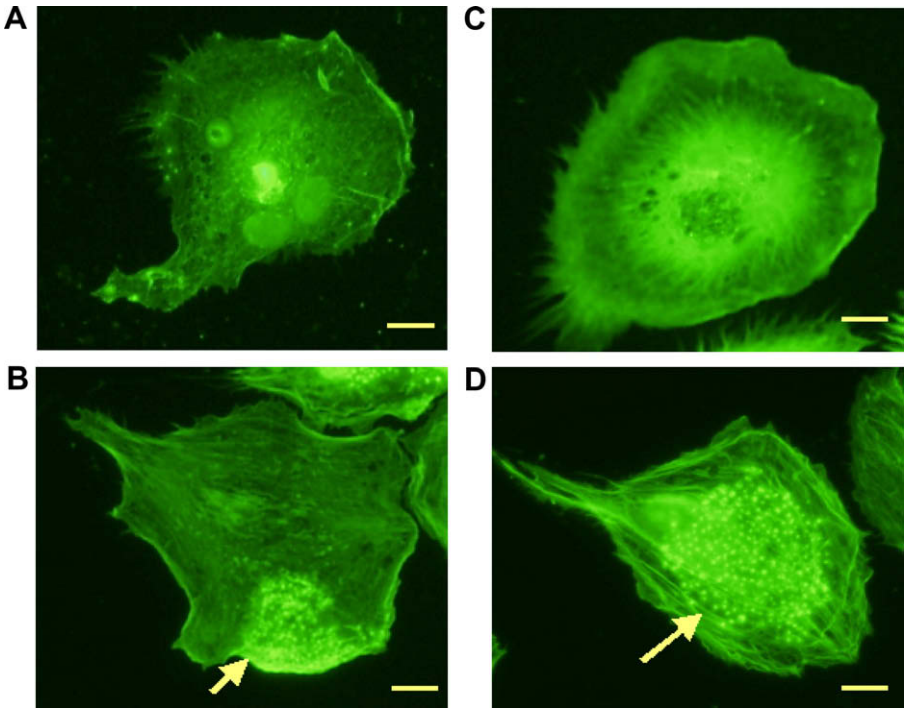

Fig. 8. The actin structure was visualized with the staining of the cells with Alexa Fluor 488-conjugated phalloidin. (A) The filamentous actin in EPC without stimulation. (B) The actin organization and lamellipodium formation at the cortex of EPC under adiponectin stimulation. (C) Rac1-siRNA blocked adiponectin-induced actin organization and lamellipodia formation in EPC. (D) Akt-siRNA did not effect the adiponectin-induced actin organization and lamellipodia formation in EPCs (scale-bar = 20 μm).

understanding of the biological functions of adiponectin and its potential clinical applications.

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